

Available online at www.sciencedirect.com

SCIENCE @ DIRECT®

Developmental Biology 290 (2006) 460–469

DEVELOPMENTAL
BIOLOGYwww.elsevier.com/locate/ydbio

Genomes & Developmental Control

Transcriptional regulation of post-aggregation genes in *Dictyostelium* by a feed-forward loop involving GBF and LagC

Negin Iranfar, Danny Fuller, William F. Loomis*

Cell and Developmental Biology, Division of Biological Sciences, University of California San Diego, La Jolla, CA 92093, USA

Received for publication 13 September 2005, revised 24 October 2005, accepted 16 November 2005

Available online 4 January 2006

Abstract

Expression profiles of developmental genes in *Dictyostelium* were determined on microarrays during development of wild type cells and mutant cells lacking either the DNA binding protein GBF or the signaling protein LagC. We found that the mutant strains developed in suspension with added cAMP expressed the pulse-induced and early adenylyl cyclase (ACA)-dependent genes, but not the later ACA-dependent, post-aggregation genes. Since expression of *lagC* itself is dependent on GBF, expression of the post-aggregation genes might be controlled only by signaling from LagC. However, expression of *lagC* in a GBF-independent manner in a *gbfA*[−] null strain did not result in expression of the post-aggregation genes. Since GBF is necessary for accumulation of LagC and both the DNA binding protein and the LagC signal transduction pathway are necessary for expression of post-aggregation genes, GBF and LagC form a feed-forward loop. Such network architecture is a common motif in diverse organisms and can act as a filter for noisy inputs. Breaking the feed-forward loop by expressing *lagC* in a GBF-independent manner in a *gbfA*⁺ strain does not significantly affect the patterns of gene expression for cells developed in suspension with added cAMP, but results in a significant delay at the mound stage and asynchronous development on solid supports. This feed-forward loop can integrate temporal information with morphological signals to ensure that post-aggregation genes are only expressed after cell contacts have been made.

© 2005 Elsevier Inc. All rights reserved.

Keywords: Transcriptional profiling; Feed-forward loop; *Dictyostelium* development; DNA binding protein; Adhesion signaling; Post-aggregative genes

Introduction

Development proceeds in stages where the initial cells set up the conditions necessary for differentiation of later cells (Gerhart and Kirschner, 1997; Eichenberger et al., 2004; Loomis et al., 1976). Multiple dependent pathways that vary from one cell lineage to the next are usually formed soon after fertilization in metazoans making it necessary to analyze regulation at the single cell level. In the simpler developmental system of the social amoeba *Dictyostelium discoideum*, synchronous development of millions of cells can be followed at the molecular level using microarrays (Van Driessche et al., 2002; Iranfar et al., 2003). Only two major cell types, prestalk and prespore cells, arise as the cells aggregate and these can be isolated in sufficient quantity for expression profiling (Iranfar et al., 2001). This system is amenable to detailed analyses of

the circuits that control and gate progress between the stages of development.

During the first 6 h of development, *Dictyostelium* cells acquire the ability to produce pulses of extracellular cAMP and synthesize the components necessary to respond chemotactically to cAMP such that they can aggregate (Parent and Devreotes, 1996). Initial expression of several aggregation stage genes including the adenylyl cyclase, ACA, the cAMP receptor, CAR1, the G-protein, Gα2, and the cAMP phosphodiesterase, PdsA, is independent of cAMP signaling but later genes are only expressed if the cells are exposed to cAMP pulses (Firtel, 1996; Iranfar et al., 2003; Kimmel and Firtel, 2004). Binding of cAMP to CAR1 leads to activation of ACA such that the levels of cAMP oscillate with a periodicity of about 7 min (Gerisch and Wick, 1975; Maeda et al., 2003). As the cells enter mounds, the external levels of cAMP increase and the oscillations are dampened. Genes expressed at that stage respond to constant high levels of extracellular cAMP (Firtel, 1996). Unlike the earlier genes, expression of these genes is dependent on the aggregation

* Corresponding author. Fax: +1 858 822 2094.

E-mail address: wloomis@ucsd.edu (W.F. Loomis).

stage adenylyl cyclase, ACA, even when supplied with exogenous cAMP (Iranfar et al., 2003).

Cell type specific genes are expressed at the mound stage and throughout the slug stage as the cell types sort out such that prestalk cells are at the anterior and prespore cells are at the posterior. During the culmination stage, prestalk cells construct a cellulose encased stalk and the prespore cells encapsulate to form spores when they reach the top. The transcriptional profiles change significantly from one stage to the next as well as between cell types as they take on new physiological roles (Iranfar et al., 2001, 2003; Van Driessche et al., 2002).

A DNA binding protein, GBF, has been shown to be essential for transcription of several post-aggregation genes during *Dictyostelium* development (Schnitzler et al., 1994). It has been called a molecular switch since it is essential for progression beyond the loose aggregate stage (Firtel, 1996). However, one of the GBF-dependent genes, *lagC*, is itself a signaling component necessary for expression of some of the post-aggregation genes (Brown and Firtel, 2001; Dynes et al., 1994). Mutant strains in which either *gbfA* or *lagC* is disrupted are blocked at the loose aggregate stage and do not express the post-aggregation genes *ecmA* or *cotC* (Schnitzler et al., 1994; Dynes et al., 1994). This shared phenotype might result from a dependent causal pathway in which GBF is responsible for expression of *lagC* and *LagC* is essential for progression beyond the loose aggregate stage. *LagC*-dependent intercellular signaling appears to control expression of the prestalk gene *ecmA* and the prespore gene *cotC* since neither is expressed in *lagC*[−] cells developed as pure populations but both are expressed in *lagC*[−] cells when they are mixed with an excess of wild type cells and allowed to develop into chimeric structures (Dynes et al., 1994). Since more than half the cells in the chimeras have to be wild type *lagC*⁺ for the mutant cells to express the post-aggregation marker genes, we argued that the cells had to directly interact rather than producing and responding to a secreted intercellular signal. Subsequently, we showed that *lagC* encodes a glycoprotein of 150 kDa (gp150) that is present on the surface of cells in loose aggregates and plays an essential role in EDTA-resistant adhesion at that stage (Wang et al., 2000). It appears to function by forming heterodimers that hold the cells together. Such a mechanism would be consistent with signaling in chimeras where *LagC* molecules on the surface of wild type cells could interact with their partners on the *lagC*[−] mutant cells to elicit a non-cell-autonomous response much as the integrins do in mammalian cells (Hynes, 2002). The amino acid sequence of *LagC* shows that it has an N-terminal sequence for secretion, a long extracellular domain and a transmembrane region near the C-terminus that could anchor it in the membrane (Dynes et al., 1994). Only a short C-terminal sequence is predicted to be cytoplasmic. The signal transduction pathway emanating from *LagC* is unknown.

Many post-aggregation genes such as those that encode the spore coat proteins, *cotB*, *cotC*, and *cotD*, are induced in shaken suspension cells by continuous high levels of cAMP. Expression is dependent on *cis*-regulatory regions that contain motifs of 6 to 8 bases with relatively high G/C (Datta and

Firtel, 1987; Pears and Williams, 1987, 1988; Hjorth et al., 1990; Fosnaugh and Loomis, 1991, 1993). GBF binds to these motifs in gel-shift assays but also binds similar regions that are present upstream of genes with quite different patterns of expression (Ceccarelli et al., 1992). Although the regions all have a relatively high G/C portion, no strict consensus sequence could be defined (Hjorth et al., 1990). It was suggested that GBF plays a stimulatory role in conjunction with other factors that determine temporal and cell type expression (Schnitzler et al., 1994).

The cAMP-dependent protein kinase PKA plays multiple roles in the development of *Dictyostelium* (Simon et al., 1992; Loomis, 1998). Cells in which the gene encoding the catalytic subunit is disrupted or are engineered to constitutively express a dominant negative inhibitor (Rm) fail to aggregate or express developmental genes (Mann et al., 1992, 1997; Harwood et al., 1992). Cells have also been engineered to express the dominant negative inhibitor uniquely in prespore cells after aggregation by driving Rm with the control region of the prespore specific gene *pspA* (Hopper et al., 1993a,b). These cells proceed through morphogenesis to form a well proportioned fruiting body but very few viable spores. They express prespore genes such as *cotC* and *cotD* for a brief period at the tipped aggregate stage but not thereafter as a consequence of the accumulation of the dominant negative inhibitor of PKA (Hopper et al., 1993a,b). In wild type cells, GBF binds to the high G/C regulatory regions of these genes but this binding activity is lost after the tipped aggregate stage in the *pspA*:[−]Rm cells (Hopper et al., 1995). It appears that, either directly or indirectly, PKA activity is essential for GBF function on these genes.

Constitutive PKA activity can bypass the need for cAMP pulses or the requirement for ACA activity for the early groups of genes (Iranfar et al., 2003). It appears that PKA mediates the signal from internal cAMP to expression of the early genes. Expression of the gene encoding ACA is dependent on the DNA binding protein CbfA and occurs only after development has been initiated by removal of the exogenous food source (Winckler et al., 2004). However, once the components for expression and response to cAMP are in place, it is further induced by pulses of cAMP in a manner that is independent of CbfA. Cells developing in suspension will express certain genes even in the absence of ACA due to disruption of its gene, *acaA*, if they are given exogenous pulses of cAMP. However, other genes have an absolute requirement for ACA (Iranfar et al., 2003). Cells in which ACA is functional express many post-aggregation genes when developed in suspension with added cAMP but do not express genes that are induced late in the slug stage or during culmination in cells developed on a solid support where they can make fruiting bodies. Many of the late genes are controlled by the DNA binding protein SrfA (Escalante et al., 2004). These developmental markers appear to require conditions and signals only found in the multicellular structures formed on solid supports. While these patterns of gene expression are consistent with a dependent sequence of developmental stages (Loomis et al., 1976), further dissection of the causal connections may indicate more complex arrangements such as cooperative, bi-furcating, or feed-forward loops.

Microarrays carrying 6450 cDNA targets have been used to recognize genes that are expressed at specific developmental stages under various conditions (Van Driessche et al., 2002; Iranfar et al., 2003; Escalante et al., 2004). About 250 genes increase 3-fold or more during development of wild type strains but not in *gbfA*[−] mutant strains unless they are developed together with at least 5% wild type cells or given exogenous pulses of cAMP (Winckler et al., 2004). Expression of this set of genes provides robust markers for the stages of development. We have constructed microarrays carrying oligonucleotides that recognize these developmental genes as well as oligonucleotide probes for a set of ~300 genes that are repressed shortly after the initiation of development (Mendoza et al., 2005). Where possible, we designed probes for two independent regions of each of the 600 mRNAs. Using these oligomicroarrays, we have characterized expression profiles in mutant strains in which either *gbfA* or *lagC* is disrupted as well as strains that express these genes under the control of the constitutive regulatory region of actin 15 (Knecht et al., 1986). We find that both GBF and LagC are necessary for expression of a set of 15 post-aggregation marker genes and that constitutive expression of *lagC* in a *gbfA*[−] null mutant does not bypass the defect. Since GBF is required for expression of *lagC* as well as the set of marker genes and that the signal transduction pathway emanating from LagC is also required for expression of the marker genes, these components define a coherent feed-forward loop with 3 positive signals (Milo et al., 2002). Feed-forward loops have been found to be prevalent among network motifs in *Escherichia coli* and *Saccharomyces cerevisiae*. A hierarchical cascade of feed-forward loops appears to drive the transcriptional program leading to sporulation in *Bacillus subtilis* (Eichenberger et al., 2004). With appropriate parameters, a coherent feed-forward loop can act as a filter of noisy inputs, such as can be expected during multicellular development (Mangan et al., 2003). Although the elements of the LagC pathway are presently unknown and some might be GBF-dependent, the fact that GBF binds to essential *cis*-acting sites of the post-aggregation genes, argues that this transcription factor and the signaling pathway can be formally considered a feed-forward loop.

Materials and methods

Strains, growth, development, and preparation of RNA

Strains AX4 (wild type), TL198 (*gbfA*[−]), and AK127 (*lagC*[−]) have been previously described (Knecht et al., 1986; Schnitzler et al., 1994; Kuspa and Loomis, 1992; Dynes et al., 1994). AK127 was transformed with a construct carrying actin15 driven *gbfA* gene and strain TL168 (*lagC*[−] *gbfA*^{OE}) selected for G418 resistance. TL198 was transformed with a construct carrying actin15 driven *lagC* gene and strain TL188 (*gbfA*[−]/*lagC*^{OE}) selected for G418 resistance. AK127 was transformed with the same construct to generate strain TL190 (*lagC*[−] *lagC*^{OE}). Cells were grown axenically in HL5 and collected while in the exponential phase of growth (Sussman, 1987).

For development in suspension, exponentially growing cells were collected, washed, and resuspended in 100 ml of 20 mM Na⁺ K⁺ phosphate buffer pH 6.5 at 10⁷ cells/ml and rapidly shaken at 125 rpm on a rotary shaker. To simulate normal signaling, cells were given 30 nM pulses of cAMP at 6 min periods from 2 to 6 h followed by addition of 300 μM cAMP

at 2 h intervals (Iranfar et al., 2003). At each time point 5 × 10⁷ cells were collected, pelleted, and dissolved in Trizol® reagent (Gibco/BRL) for preparation of RNA. For development on solid supports, cells were collected, washed, and deposited on nitrocellulose filters on buffer-saturated pads and allowed to develop synchronously at 22°C (Sussman, 1987).

Preparation of oligoarrays and expression analyses

Microarrays carrying unique 50 bp oligonucleotides for 600 developmental genes were prepared as previously described (Mendoza et al., 2005). Where possible, we designed probes for two independent regions of each of the 600 mRNAs to allow internal comparisons and averaging. The list of developmental genes and the microarray data for each gene in the time courses for each strain are available for downloading at <http://www.biology.ucsd.edu/loomis-cgi/microarray/gbf-lagc-suppl.html>.

DNA probes were prepared from total RNA collected at 2 h intervals as well as from time-averaged reference RNA as previously described (Iranfar et al., 2003). Superscript II DNA polymerase (Invitrogen, Carlsbad, CA) was used to incorporate either Cy-5 or Cy-3 conjugated dCTP (Amersham, St. Louis, MO) into DNA. Following incubation at 42°C for 3 h, unincorporated dyes were removed using microcon-30 columns (Millipore, Burlington, MA) with 3 washes with 450 μl TE buffer before drying and resuspending in 5× SSC, 0.3% SDS, 25% formamide. Labeled probes were mixed and hybridized at 42°C to the microarrays for 6 to 12 h. Dyes for the sample and reference probes were interchanged in different experiments.

Probed microarrays were analyzed in an Axon Genepix 4000B scanner and the measurement processes with the associated software. Total Cy3 signal was normalized to total Cy5 signal after background subtraction to allow independent slides to be compared. The ratios of Cy3/Cy5 for individual genes were then calculated. Each sample was hybridized to two or more microarrays which carried the oligonucleotides in duplicate. Each developmental time course was repeated at least twice. Mean values were used for subsequent analyses and are available at <http://www.biology.ucsd.edu/loomis-cgi/microarray/gbf-lagc-suppl.html>. Values were normalized to 1 at the start of each experiment. Statistical analyses were previously described (Iranfar et al., 2003).

Bar graphs of the expression profiles represent the average fold change as blue (<2), yellow (2–4), tan (4–6), brown (6–8), reddish brown (8–10), and red (>10). Profiles that had only a single point above 3-fold are presented in blue. Statistical reliability can be judged by comparing the results from the independent strains.

Northern analyses

Gel-separated RNA was transferred to nylon membranes (Osmonics, Inc., Westborough, MA) and Northern blots were probed as previously described (Iranfar et al., 2001). ³²P-labeled probes were generated by random hexamer labeling of DNA fragments.

Results

Post-aggregation genes

To simulate normal signaling, cells developing in suspension were given 30 nM pulses of cAMP at 6 min periods from 2 to 6 h followed by addition of 300 μM cAMP at 2 h intervals (Iranfar et al., 2003). RNA samples were collected at 2 h intervals from wild type, *gbfA*[−], and *lagC*[−] cells developed in this manner and analyzed on microarrays. Expression profiles for wild type cells determined with oligonucleotide probes matched those previously determined with microarrays carrying 6345 cDNAs (Iranfar et al., 2003). With the oligoarrays, we focused on a set of 3 genes that are pulse-independent, 11 genes that are pulse-dependent but are expressed in an *acaA*[−] strain and 28 genes that are expressed in pulsed wild type cells

but not in pulsed *acaA*[−] cells (Iranfar et al., 2003). mRNAs from about half of the ACA-dependent genes start to accumulate after 2–4 h while the rest start to accumulate steadily after about 8 h (Fig. 1). Strains in which either *gbfA* or *lagC* is disrupted express all of the pulse-independent and pulse-dependent genes except for *lagC* and express the early group of ACA-dependent genes but none of the later ACA-dependent genes (Fig. 1). These results confirm the observations of Brown and Firtel (2001) that *lagC* is not expressed in *gbfA*[−] null cells. Of course, *lagC* is not expressed in *lagC*[−] null cells since it is disrupted. The microarray results also confirm that the prespore gene *cotC* is not expressed in either *gbfA*[−] null cells or *lagC*[−] null cells (Schnitzler et al., 1994; Dynes et al., 1994). Moreover, the microarray data show that all of the early marker genes expressed in *gbfA*[−] are expressed in *lagC*[−] null cells and that none of the GBF-dependent marker genes are expressed in *lagC*[−] null cells. About half of the GBF-dependent genes are prespore specific and half are prestalk specific. Since it has been shown that GBF accumulates normally in pulsed *lagC*[−] null cells (Brown and Firtel, 2001), it appears that expression of these post-aggregation genes is dependent on both GBF and LagC. Since expression

of *lagC* is GBF-dependent, expression of later genes could depend only on a LagC-dependent signal.

GBF-independent expression of *lagC*

To determine whether the GBF-dependent genes fail to be expressed in *gbfA*[−] cells due to the lack of expression of LagC, we placed the *lagC* gene under the control of the actin 15 regulatory region. Actin 15 is expressed at high levels in growing cells and throughout the first 16 h of development (Knecht et al., 1986). However, except for *lagC* itself, expression of act15:*lagC* in a *gbfA*[−] null strain did not result in expression of the GBF-dependent genes (Fig. 2). The pulse-independent, pulse-dependent, and ACA-dependent genes were all expressed normally but none of the later genes were expressed. Introduction of the act15:*lagC* construct into a *lagC*[−] strain resulted in expression of the GBF-dependent genes, as expected, since these cells express both *gbfA* and *lagC* (Fig. 2).

Expression of an act15:*gbfA* construct introduced into *lagC*[−] null cells did not result in expression of the GBF-dependent genes since these cells lack LagC (Fig. 2). All the

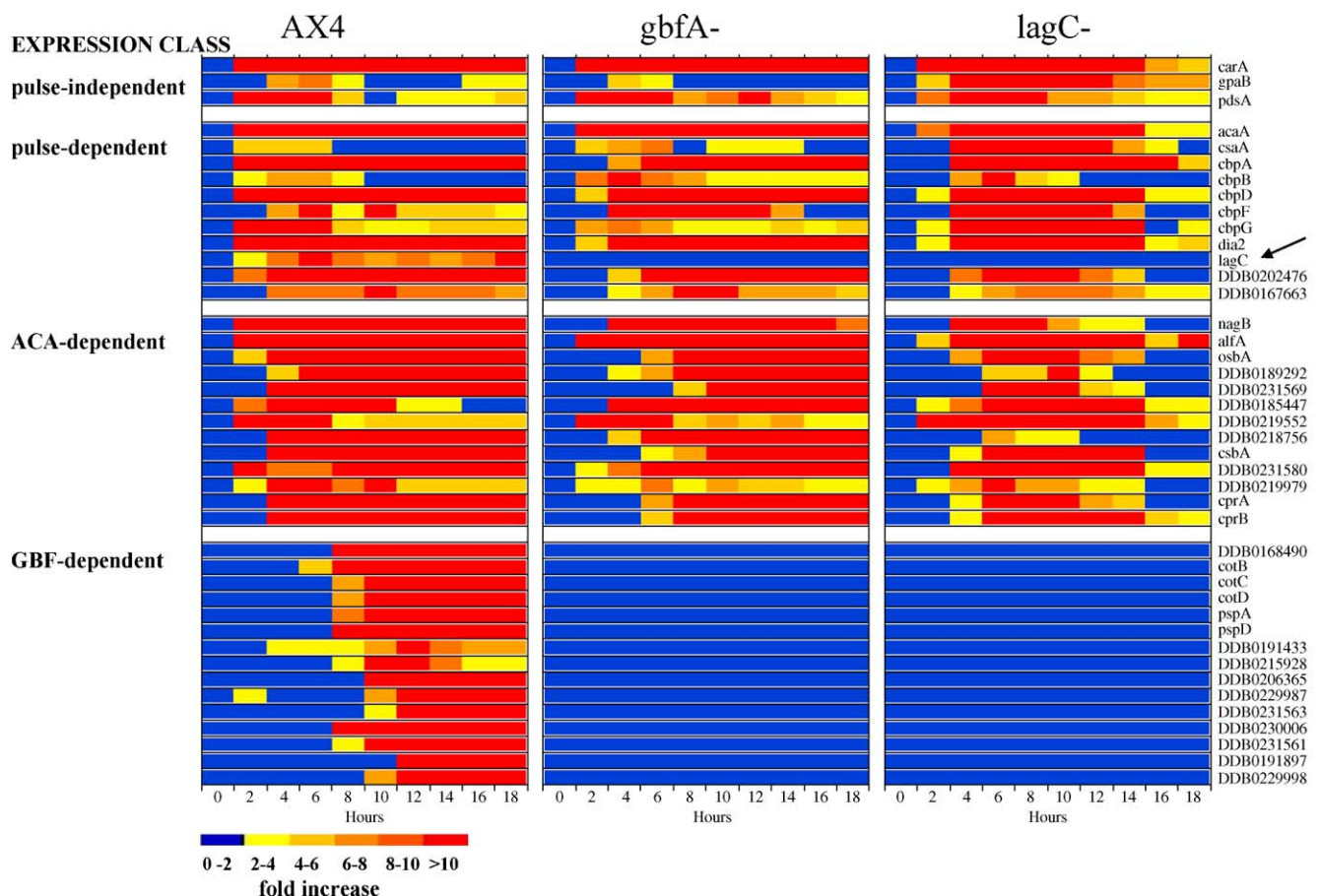


Fig. 1. Expression profiles of early and post-aggregation genes. AX4 (wild type), *gbfA*[−], and *lagC*[−] cells were developed in suspension and given 30 nM cAMP pulses at 6 min intervals between 2 and 6 h followed by addition of 300 μM cAMP. Samples were collected every 2 h and analyzed on oligomicroarrays. Each developmental time course was repeated at least twice and the mean values were normalized to 1 at the start of each experiment. Bar graphs of the expression profiles represent the average fold change as blue (<2), yellow (2–4), tan (4–6), brown (6–8), reddish brown (8–10), and red (>10). The expression pattern of *lagC* is indicated by an arrow. The expression classes are indicated. Values for each target are available at <http://www.biology.ucsd.edu/loomis-cgi/microarray/gbf-lagc-suppl.html>.

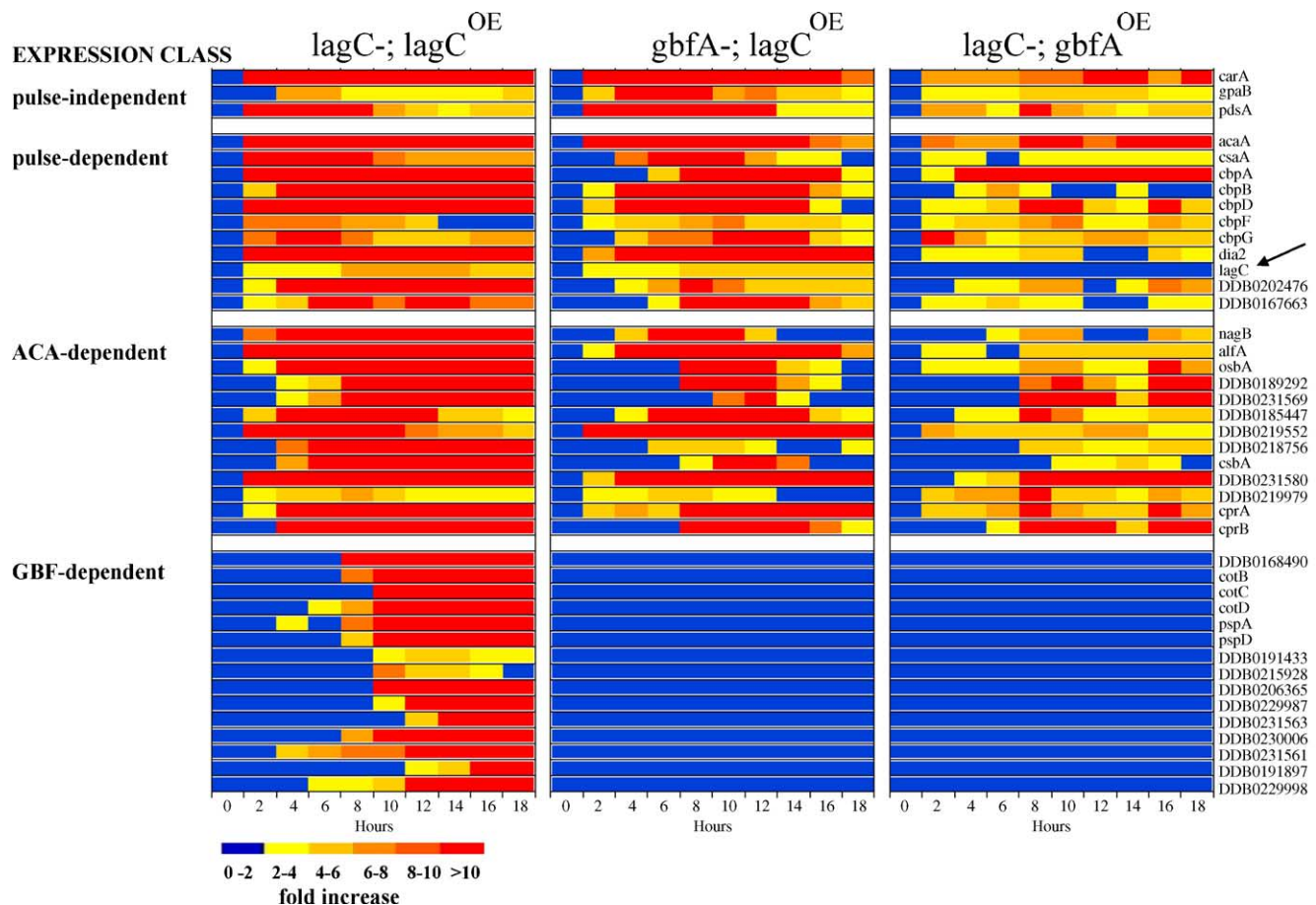


Fig. 2. Coordinately regulated genes in cells constitutively expressing either *lagC* or *gbfA*. Cells were developed in suspension, pulsed between 2 and 6 h followed by addition of 300 μ M cAMP. Samples were prepared for microarray analyses and the results displayed as in Fig. 1. The expression pattern of *lagC* is indicated by an arrow. The expression classes are indicated.

other early marker genes were expressed normally with no evidence for precocious induction confirming the conclusions of Schnitzler et al. (1994) that were based on a smaller number of marker genes. When plated on solid supports, these *lagC*[−] *gbfA*^{OE} cells developed up to the loose aggregate stage but failed to proceed further in morphogenesis (data not shown). Only when spread on agar plates did some of the loose aggregates proceed to form standing fingers which slowly progressed to form a few small fruiting bodies after more than 40 h of development. Sukumaran et al. (1998) suggested that GBF overexpression in *lagC*[−] null cells could rescue the signaling defects because they found a few migrating slugs. However, morphogenesis is clearly aberrant and the expression analyses clearly indicate that overexpression of GBF in a *lagC*[−] strain does not overcome the block to expression of the post-aggregation genes (Fig. 2).

Northern analyses

To confirm the critical results from the microarray analyses, we electrophoretically separated the RNA samples on agarose gels and transferred them to Nitran for Northern analyses. The results from probing with *lagC* and *cotB* were completely consistent with the microarray results (Fig. 3). *lagC* mRNA

accumulated in wild type cells by 4 h of development and then tapered off. It was barely detectable in *gbfA*[−] and absent in *lagC*[−] and *lagC*[−] *gbfA*^{OE} cells. *lagC* mRNA was present in vegetative *gbfA*[−] *lagC*^{OE} cells (*T* = 0) as expected from being controlled by the regulatory region of actin 15. This was not apparent in the microarray data shown in Fig. 2 because it was normalized to *T* = 0 for ease of interpretation; however, the unnormalized levels of *lagC* were higher in vegetative cells of both *gbfA*[−] *lagC*^{OE} and *lagC*[−] *lagC*^{OE} cells. The microarray results show that it accumulated at least 4-fold by 12 h which is consistent with the Northern results.

mRNA for the prespore gene *cotB* failed to accumulate in any of the strains lacking either GBF or LagC but accumulated normally when *lagC*[−] was complemented by act15::*lagC* (Fig. 3). The Northern results confirm the expression patterns determined using microarrays.

GBF-dependent genes

Some of the genes from the 6450 cDNAs microarrays chosen as developmental markers have been used for many years as aggregation stage and post-aggregation genes in Northern analyses. These include *lagC*, *cotB*, *cotC*, *cotD*, *pspA*, *pspD*, and *D7* (Dynes et al., 1994; Fosnaugh and Loomis, 1991;

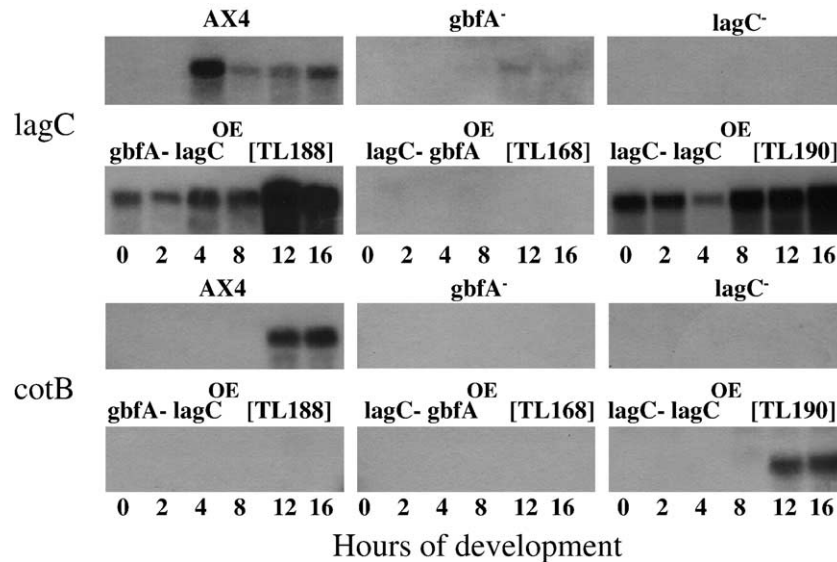


Fig. 3. Northern analyses. RNA samples from the various strains that were prepared for microarray analyses at the times indicated. 15 μ g of total RNA was size fractionated by gel electrophoresis, transferred to nylon membranes, and hybridized to probes for *lagC* or *cotB*.

Agarwal et al., 1994; Early et al., 1988; Firtel, 1996). Many of these genes encode components of the spore coat and are only expressed in prespore cells where their protein products are localized in prespore vesicles (Alexander et al., 2003). The cell type specificity of each of the GBF-dependent genes was previously determined by characterization of RNA from purified prespore and prestalk cells and confirmed by in situ hybridization (Maeda et al., 2003; Maruo et al., 2004). Of the 16 genes on the oligoarrays that are GBF-dependent, 7 are prespore specific and 7 are prestalk specific. Only two (*lagC* and DDB0215928) are expressed in both cell types (Table 1). Three of the prestalk specific GBF-dependent genes (DDB0168490; DDB0231563; DDB0191897) encode related proteins of 88 to 93 amino acids that are predicted to form coiled coils. Another three of the prestalk specific GBF-dependent genes (DDB0229987; DDB0231561; DDB0229998) encode almost identical small proteins of 57 amino acids. There are 13 such genes encoding exactly 57 amino acids with almost identical sequences. Eleven of them are found in a tandem array cluster on chromosome 4 (Eichinger et al., 2005). The sequences of the 50 bp oligonucleotides for the three genes on the microarrays (DDB0229987; DDB0231561; DDB0229998) differ by only a few nucleotides from the mRNA sequences from many of these genes possibly resulting in cross-hybridization.

GBF has two central zinc-fingers that are sufficient for it to bind to several different motifs in gel-shift experiments (Hjorth et al., 1990; Brown and Firtel, 2001). Moreover, expression of a construct with only a single zinc finger was found to be sufficient for complementation of a *gbfA*⁻ null strain (Brown and Firtel, 2001). A single zinc finger can only recognize a sequence of 3 bases (Kaplan et al., 2005). GBF recognizes motifs containing CACAC or ACCC as well as their reverse complements (Hjorth et al., 1990). Such motifs are found in the 500 bp regions upstream of about a third of the genes in the *Dictyostelium* genome and so cannot provide much specificity on their own. However, when the upstream regions of the

GBF-dependent genes on the microarrays were inspected, clusters of such sites were seen usually within 100 bp of each other (Table 1). Such sites where GBF could bind might function cooperatively together with other transcription factors. Both the prespore and prestalk specific GBF-dependent genes have multiple sites where GBF could bind (Table 1).

Discussion

The genes encoding the DNA binding protein GBF and the cell adhesion protein LagC are both expressed during the first 4 h of development in suspension when the cells are given cAMP pulses (Schnitzler et al., 1994, 1995; Dynes et al., 1994). When developed on solid supports, *gbfA* mRNA is first observed at 4 h while *lagC* mRNA is first observed at 8 h, consistent with the fact that expression of *lagC* is dependent on GBF while expression of *gbfA* is independent of LagC (Brown and Firtel, 2001). When developed in suspension, the only gene on the microarrays expressed in the first 4 h that was found to be GBF-dependent was *lagC*. All other GBF-dependent genes are expressed several hours later and are also dependent on LagC (Fig. 1). GBF has been shown in gel-shift experiments to recognize essential *cis*-acting sites for many of these genes and LagC has been found to mediate an intercellular signaling pathway essential for expression of these genes. Since expression of *lagC* by a GBF-independent regulatory region (*act15*) does not result in expression of the post-aggregation genes in a *gbfA*⁻ null strain, these genes are formally controlled by a feed-forward loop containing GBF and LagC (Fig. 4). Consistent with the dual requirement, overexpression of *gbfA* in a *lagC*⁻ null strain does not result in expression of the post-aggregation genes.

The feed-forward loop is no longer functioning in cells with wild type *gbfA* when *lagC* is expressed under the control of the GBF-independent regulatory region of actin 15 (Fig. 4). The *lagC*⁻ strain expressing *act15::lagC* has wild type GBF and

Table 1
GBF-dependent genes

Gene	Locus/cDNA	Product/localization	Cell type	Motif	Position bp to ATG
DDB0214927	lagC	GP150 surface	Non-specific	CACAC	330
				AGGGGT	380
				ACCCC	360
DDB0168490	SSJ767	88 aa	Prestalk	CACAC	430
				CACAC	200
				GTGTGT	308
				TGGGGT	320
				ACCCCCA	180
DDB0185091	cotB (SP70)	Spore coat (PSV)	Prespore	CACCCACACAC	490
				CACAC	430
				GACGGGT	370
				ACCCA	420
DDB0185189	cotC (SP60)	Spore coat (PSV)	Prespore	CACAC	475
				CACAC	450
				TCCCA	482
DDB0191431	cotD (SP75)	Spore coat (PSV)	Prespore	CACACACAC	385
				CACAC	375
				CACACA	332
				ACCCA	350
DDB0191129	pspA	Unknown	Prespore	CACAC	341
				TTGGGA	475
				ACCCA	223
DDB0191310	pspD (SP87)	Spore coat (PSV)	Prespore	ACACA	298
				GTGTGT	632
				TGGGT	408
DDB0191433	SSD275 (D7)	PSV	Prespore	CACACAC	490
				CACACACAC	446
				GTGTG	345
				AGGGGGT	320
DDB0215928	SLH423	Unknown	Non-specific	CACAC	817
				GTGGGT	963
				CACCCACA	825
				CACCCA	910
DDB0206365	SSB240	Unknown	Prespore	CACAC	510
				CACAC	445
				CACCCA	485
DDB0229987	SSF295	57 aa	Prestalk	CACAC	172
				CACAC	178
				GTGGGT	320
DDB0231563	SSL853	93 aa	Prestalk	CACAC	418
				GTGTGTG	346
				GGGGGT	246
DDB0230006	SSG159	Unknown	Prestalk	CACAC	240
				CACACAC	230
				CACAC	205
				ACCCA	442
DDB0231561	SSF823	57 aa	Prestalk	CACA	361
				GTGTGTGT	410
				GGGGGT	250
				ACCCT	303
DDB0191897	SSM416	93 aa	Prestalk	CACACAC	925
				GTGTGT	850
				ACCCT	720
DDB0229998	SSL349	57 aa	Prestalk	CACACACA	275
				CACACA	265
				GTGGGT	241
				CCCCA	286

expresses post-aggregation genes in essentially the same manner as wild type AX4 cells (see Figs. 1 and 2). Thus, there appears to be no significant consequences when the feed-forward loop is converted to a convergent network. However,

these analyses were carried out under conditions designed to minimize developmental variability. Exponentially growing cells were washed free of growth medium and immediately suspended in buffer and continuously shaken. Pulses of cAMP

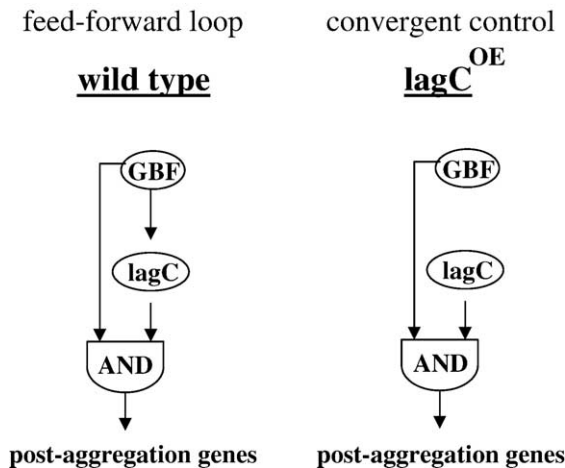


Fig. 4. Network architecture. A feed-forward loop controls expression of post-aggregation genes in wild type cells but not in cells expressing *lagC* under the control of a GBF-independent regulatory region (actin 15). In wild type cells, GBF is responsible for expression of *lagC* as well as post-aggregation genes. In both wild type and act15:*lagC* cells, GBF and LagC are both required for expression of post-aggregation genes, but expression of *lagC* is independent of GBF in the act15:*lagC* cells.

were added at 6 min intervals between 2 and 6 h and then high levels of cAMP were added. Small clumps of cells formed after 6 h in suspension but they never exceeded 100 cells. When the *lagC⁻* act15:*lagC* cells were allowed to develop on solid supports, they aggregated and formed fruiting bodies by 28 h, but development was much less synchronous and the resulting fruiting bodies were smaller and less regular than seen with wild type cells (data not shown). We had previously found that expression of act15:*lagC* in a *lagC⁻* strain overcame the block at the loose aggregate stage but that the cells remained at the tight aggregate stage for 2–6 h longer than wild type cells (Dynes et al., 1994). Although the morphological abnormalities of the act15:*lagC lagC⁻* strain might be the result of alterations in the pattern of expression of *lagC*, they might also be an indication that the lack of the feed-forward loop is detrimental to development.

The GBF/LagC feed-forward loop can integrate temporal signals with morphological signals to ensure that post-aggregation genes are only expressed after cells have aggregated and formed LagC-dependent cell–cell contacts. At least in prespore cells, GBF activity in gel-shift experiments is dependent on PKA activity which controls developmental timing (Hopper et al., 1995; Loomis, 1998). Overexpression of the catalytic subunit of PKA results in rapid development and the ability of cells to form spores in submerged conditions (Anjard et al., 1992; Hopper et al., 1993a,b). However, overexpression of the PKA catalytic subunit in a *lagC⁻* strain does not result in expression of the post-aggregation genes (data not shown). While PKA activity is responsible for the timing of expression of post-aggregation genes, it is not sufficient in the absence of LagC. Likewise, precocious expression of *lagC*, such as occurs in the *lagC⁻ lagC^{OE}* cells (Fig. 3), does not result in precocious expression of the post-aggregation genes. Timing depends on more than the presence of LagC.

The signal transduction pathway that is activated by LagC is presently unknown. It appears to depend on cell–cell contact since mixtures of equal numbers of *lagC⁻* and wild type cells develop abnormally while mixtures with 75% wild type cells develop well (Dynes et al., 1994). When wild type cells are developed in suspension and treated with cAMP, cell–cell contacts can form in the small clumps of cells and this seems to be sufficient for expression of post-aggregation genes. If other components of the LagC signal transduction pathway are also GBF-dependent, it would not change the formal designation of GBF/LagC as a feed-forward loop, but would only reinforce it.

The two zinc-fingers of GBF appear to be only sufficient for it to be a low affinity/low specificity factor that must act in concert with other factors (Brown and Firtel, 2001). However, there are clusters of the GBF motifs CACAC and ACCCA within 200 bp of each other upstream of the GBF-dependent post-aggregation genes. In all but two cases, these clusters are found in the proximal 500 bp (Table 1). About 7% of all genes in *Dictyostelium* have clusters of 3 or more such motifs within 200 bp of each other in the 500 bp upstream region. However, these motifs are strongly enriched in the upstream regions of the GBF-dependent genes.

Seven of the GBF-dependent marker genes on the microarrays are only expressed in prestalk cells and 7 are only expressed in prespore cells. Clearly, the cell type specificity of expression of these genes is determined by other factors that respond to the conditions in the specific cell types. Nevertheless, they are all coordinately regulated and as such define a set of marker genes for the post-aggregation stage of development.

Acknowledgments

We thank Professor Richard Firtel for the *ghfA⁻* null strain, Samuel Payne for genomic motif searches and Professor Gad Shaulsky for insightful advice. This work was supported by a grant from the NIH (GM62350).

References

- Agarwal, A., Sloger, M.S., Oyama, M., Blumberg, D.D., 1994. Analysis of a novel cyclic AMP inducible prespore gene in *Dictyostelium discoideum*: evidence for different patterns of cAMP regulation. *Differentiation* 57, 151–162.
- Alexander, S., Srinivasan, S., Alexander, H., 2003. Proteomics opens doors to the mechanisms of developmentally regulated secretion. *Mol. Cell. Proteomics* 2, 1156–1163.
- Anjard, C., Pinaud, S., Kay, R.R., Reymond, C.D., 1992. Overexpression of Dd PK2 protein kinase causes rapid development and affects the intracellular cAMP pathway of *Dictyostelium*. *Development* 115, 785–790.
- Brown, J.M., Firtel, R.A., 2001. Functional and regulatory analysis of the *Dictyostelium* G-box binding factor. *Dev. Biol.* 234, 521–534.
- Ceccarelli, A., Mahbubani, H.J., Insall, R., Schnitzler, G., Firtel, R.A., Williams, J.G., 1992. A G-rich sequence element common to *Dictyostelium* genes which differ radically in their patterns of expression. *Dev. Biol.* 152, 188–193.
- Datta, S., Firtel, R.A., 1987. Identification of the sequences controlling cyclic AMP regulation and cell-type-specific expression of a prestalk-specific gene in *Dictyostelium discoideum*. *Mol. Cell. Biol.* 7, 149–159.

- Dynes, J.L., Clark, A.M., Shaulsky, G., Kuspa, A., Loomis, W.F., Firtel, R.A., 1994. LagC is required for cell–cell interactions that are essential for cell-type differentiation in *Dictyostelium*. *Genes Dev.* 8, 948–958.
- Early, A.E., Williams, J.G., Meyer, H.E., Por, S.B., Smith, E., Williams, K.L., Gooley, A.A., 1988. Structural characterization of *Dictyostelium discoideum* prespore-specific gene D19 and of its product, cell surface glycoprotein PsA. *Mol. Cell. Biol.* 8, 3458–3466.
- Eichenberger, P., Fujita, M., Jensen, S.T., Conlon, E.M., Rudner, D.Z., Wang, S.T., Ferguson, C., Haga, K., Sato, T., Liu, J.S., Losick, R., 2004. The program of gene transcription for a single differentiating cell type during sporulation in *Bacillus subtilis*. *PLoS Biol.* 2, 1664–1683.
- Eichinger, L., Pachebat, J., Glockner, G., Rajandream, M.-A., Sugang, R., Berriman, M., Song, J., Olsen, R., Szafranski, K., Xu, Q., Tunggal, B., Kummerfeld, S., Madera, M., Konfortov, A., Rivero, F., Bankier, A., Lehmann, R., Hamlin, N., Davies, R., Gaudet, P., Fey, P., Pilcher, K., Chen, G., Saunders, D., Soderger, E., Davis, P., Kehomou, A., Nie, X., Hall, N., Anjard, C., Hemphill, L., Bason, N., Farbrother, P., Desany, B., Just, E., Morio, T., Rost, R., Churcher, C., Cooper, J., Haydock, S., van Driessche, N., Cronin, A., Goodhead, I., Muzny, D., Mourier, T., Pain, A., Lu, M., Harper, D., Lindsay, R., Hauser, H., James, K., Quiles, M., Mohan, M., Saito, T., Buchrieser, C., Wardroper, A., Felder, M., Thangavelu, M., Johnson, D., Knights, A., Loulsegh, H., Mungall, K.M., Oliver, K., Price, C., Quail, M., Urushihara, H., Hernandez, J., Rabinowitsch, E., Steffen, D., Sanders, M., Ma, J., Kohara, Y., Sharp, S., Simmonds, M., Spiegler, S., Tivey, A., Sugano, S., White, B., Walker, D., Woodward, J., Winckler, T., Tanaka, Y., Shaulsky, G., Schleicher, M., Weinstock, G., Rosenthal, A., Cox, E., Chisholm, R., Gibbs, R., Loomis, W.F., Platzer, M., Kay, R.R., Williams, J., Dear, P., Noegel, A.A., Barrell, B., Kuspa, A., 2005. The genome of the social amoeba *Dictyostelium discoideum*. *Nature* 435, 43–57.
- Escalante, R., Iranfar, N., Sastre, L., Loomis, W.F., 2004. Identification of genes dependent on the MADS-box transcription factor SrFA in *Dictyostelium* development. *Euk. Cell* 3, 564–566.
- Firtel, R.A., 1996. Interacting signaling pathways controlling multicellular development in *Dictyostelium*. *Curr. Opin. Genet. Devel.* 6, 545–554.
- Fosnaugh, K.L., Loomis, W.F., 1991. Coordinate regulation of the spore coat genes in *Dictyostelium discoideum*. *Dev. Genet.* 12, 123–132.
- Fosnaugh, K.L., Loomis, W.F., 1993. Enhancer regions responsible for temporal and cell-type-specific expression of a spore coat gene in *Dictyostelium*. *Dev. Biol.* 157, 38–48.
- Gerhart, J., Kirschner, M., 1997. *Cells, Embryos, and Evolution*. Blackwell Science, Malden, MA.
- Gerisch, G., Wick, U., 1975. Intracellular oscillations and release of cyclic AMP from *Dictyostelium* cells. *Biochem. Biophys. Res. Commun.* 65, 364–370.
- Harwood, A.J., Hopper, N.A., Simon, M.N., Bouzid, S., Veron, M., Williams, J.G., 1992. Multiple roles for cAMP-dependent protein kinase during *Dictyostelium* development. *Dev. Biol.* 149, 90–99.
- Hjorth, A.L., Pears, C., Williams, J.G., Firtel, R.A., 1990. A developmentally regulated *trans*-acting factor recognizes dissimilar G/C-rich elements controlling a class of cAMP-inducible *Dictyostelium* genes. *Genes Dev.* 4, 419–432.
- Hopper, N.A., Anjard, C., Reymond, C.D., Williams, J.G., 1993a. Induction of terminal differentiation of *Dictyostelium* by cAMP-dependent protein kinase and opposing effects of intracellular and extracellular cAMP on stalk cell differentiation. *Development* 119, 147–154.
- Hopper, N.A., Harwood, A.J., Bouzid, S., Veron, M., Williams, J.G., 1993b. Activation of the prespore and spore cell pathway of *Dictyostelium* differentiation by cAMP-dependent protein kinase and evidence for its upstream regulation by ammonia. *EMBO J.* 12, 2459–2466.
- Hopper, N.A., Sanders, G.M., Fosnaugh, K.L., Williams, J.G., Loomis, W.F., 1995. Protein kinase A is a positive regulator of spore coat gene transcription in *Dictyostelium*. *Differentiation* 58, 183–188.
- Hynes, R.O., 2002. Integrins: bidirectional allosteric signaling machines. *Cell* 110, 673–687.
- Iranfar, N., Fuller, D., Sasik, R., Hwa, T., Laub, M., Loomis, W.F., 2001. Expression patterns of cell-type-specific genes in *Dictyostelium*. *Mol. Biol. Cell* 12, 2590–2600.
- Iranfar, N., Fuller, D., Loomis, W.F., 2003. Genome-wide expression analyses of gene regulation during early development of *Dictyostelium discoideum*. *Euk. Cell* 2, 664–670.
- Kaplan, T., Friedman, N., Margalit, H., 2005. Ab initio prediction of transcription factor targets using structural knowledge. *PLoS Computational Biol.* 1, 5–13.
- Kimmel, A.R., Firtel, R.A., 2004. Breaking symmetries: regulation of *Dictyostelium* development through chemoattractant and morphogen signal-response. *Curr. Opin. Genet. Devel.* 14, 540–549.
- Knecht, D.A., Cohen, S.M., Loomis, W.F., Lodish, H.F., 1986. Developmental regulation of *Dictyostelium Dictyostelium* actin gene fusions carried on low-copy and high-copy transformation vectors. *Mol. Cell. Biol.* 6, 3973–3983.
- Kuspa, A., Loomis, W.F., 1992. Tagging developmental genes in *Dictyostelium* by restriction enzyme-mediated integration of plasmid DNA. *Proc. Natl. Acad. Sci. U. S. A.* 89, 8803–8807.
- Loomis, W.F., 1998. Role of PKA in the timing of developmental events in *Dictyostelium* cells. *Microbiol. Mol. Biol. Rev.* 62, 684.
- Loomis, W.F., White, S., Dimond, R.L., 1976. A sequence of dependent stages in the development of *Dictyostelium discoideum*. *Dev. Biol.* 53, 171–177.
- Maeda, M., Sakamoto, H., Iranfar, N., Fuller, D.T., Ogihara, S., Morio, T., Urushihara, H., Tanaka, Y., Loomis, W.F., 2003. Changing patterns of gene expression in *Dictyostelium* prestalk cell subtypes recognized by in situ hybridization with genes from microarray analyses. *Euk. Cell* 2, 627–637.
- Mangan, S., Zaslaver, A., Alon, U., 2003. The coherent feedforward loop serves as a sign-sensitive delay element in transcription networks. *J. Mol. Biol.* 334, 197–204.
- Mann, S.K.O., Yonemoto, W.M., Taylor, S.S., Firtel, R.A., 1992. DdPK3, which plays essential roles during *Dictyostelium* development, encodes the catalytic subunit of cAMP-dependent protein kinase. *Proc. Natl. Acad. Sci. U. S. A.* 89, 10701–10705.
- Mann, S.K.O., Brown, J.M., Briscoe, C., Parent, C., Pitt, G., Devreotes, P.N., Firtel, R.A., 1997. Role of cAMP-dependent protein kinase in controlling aggregation and postaggregative development in *Dictyostelium*. *Dev. Biol.* 183, 208–221.
- Maruo, T., Sakamoto, H., Iranfar, N., Fuller, D., Morio, T., Urushihara, H., Tanaka, Y., Maeda, M., Loomis, W.F., 2004. Control of cell type proportioning in *Dictyostelium discoideum* by differentiation-inducing factor as determined by in situ hybridization. *Euk. Cell* 3, 1241–1248.
- Mendoza, M., Du, F., Iranfar, N., Tang, N., Ma, H., Loomis, W.F., Firtel, R.A., 2005. Loss of SMEK, a novel, conserved protein, suppresses *mek1* null cell polarity and chemotaxis defects. *Mol. Cell. Biol.* 25, 7839–7853.
- Milo, R., Shen-Orr, S., Itzkovitz, S., Kashtan, N., Chklovskii, D., Alon, U., 2002. Network motifs: simple building blocks of complex networks. *Science* 298, 824–827.
- Parent, C.A., Devreotes, P.N., 1996. Molecular genetics of signal transduction in *Dictyostelium*. *Annu. Rev. Biochem.* 65, 411–440.
- Pears, C.J., Williams, J.G., 1987. Identification of a DNA sequence element required for efficient expression of a developmentally regulated and cAMP-inducible gene of *Dictyostelium discoideum*. *EMBO J.* 6, 195–200.
- Pears, C.J., Williams, J.G., 1988. Multiple copies of a G-rich element upstream of a cAMP-inducible *Dictyostelium* gene are necessary but not sufficient for efficient gene expression. *Nucleic Acids Res.* 16, 8467–8486.
- Schnitzler, G.R., Fischer, W.H., Firtel, R.A., 1994. Cloning and characterization of the G-box binding factor, an essential component of the developmental switch between early and late development in *Dictyostelium*. *Genes Dev.* 8, 502–514.
- Schnitzler, G.R., Briscoe, C., Brown, J.M., Firtel, R.A., 1995. Serpentine cAMP receptors may act through a G protein-independent pathway to induce postaggregative development in *Dictyostelium*. *Cell* 81, 737–745.
- Simon, M.N., Pelegri, O., Veron, M., Kay, R.R., 1992. Mutation of protein kinase A causes heterochronic development of *Dictyostelium*. *Nature* 356, 171–172.
- Sukumaran, S., Brown, J.M., Firtel, R.A., McNally, J.G., 1998. *lagC*-null and *gbf*-null cells define key steps in the morphogenesis of *Dictyostelium* mounds. *Dev. Biol.* 200, 16–26.

- Sussman, M., 1987. Cultivation and synchronous morphogenesis of *Dictyostelium* under controlled experimental conditions. In: Spudich, J.A. (Ed.), *Methods in Cell Biology*, vol. 28. Ac. Press, Orlando, FL, pp. 9–29.
- Van Driessche, N., Shaw, C., Katoh, M., Morio, T., Sucgang, R., Ibarra, M., Kuwayama, H., Saito, T., Urushihara, H., Maeda, M., Takeuchi, I., Ochiai, H., Eaton, W., Tollett, J., Halter, J., Kuspa, A., Tanaka, Y., Shaulsky, G., 2002. A transcriptional profile of multicellular development in *Dictyostelium discoideum*. *Development* 129, 1543–1552.
- Wang, J., Huo, L., Awrey, D., Loomis, W.F., Firtel, R.A., Siu, C.-H., 2000. Membrane glycoprotein gp150 is encoded by the *lagC* gene and mediates cell–cell adhesion via heterophilic binding during *Dictyostelium* development. *Dev. Biol.* 227, 734–745.
- Winckler, T., Iranfar, N., Beck, P., Jennes, I., Siol, O., Baik, U., Loomis, W.F., Dingermann, T., 2004. CbfA, the C-module DNA-binding factor, plays an essential role in the initiation of *Dictyostelium discoideum* development. *Euk. Cell* 3, 1349–1358.